



Duloxetine metabolism in the presence of Cytochrome P450 inhibitors

Astyia Z. Franken-Golden, Dr. Myoung E. Lee, Dr. Emily F. Ruff

Department of Chemistry, Winona State University, Winona, Minnesota



Abstract

Cytochrome P450 (CYP) enzymes are important for drug metabolism. They chemically modify drugs to make them more soluble, which allows the drugs to be excreted from the body. Of the 30 CYP enzymes, 6 (CYP1A2, 2C9, 2C19, 2D6, 2E1, 3A4/5) are of clinical interest because these enzymes are the key players in drug metabolism. Differences in genetics lead to individuals of the same species expressing different CYP enzymes. The different combination of CYP enzymes may affect the rate of drug metabolism or produce metabolites that may cause adverse side effects. Predicting drug metabolites and drug-drug interactions is important when designing and prescribing new drugs because other drugs can act as CYP activators or inhibitors. Duloxetine is an antidepressant mainly metabolized by CYP1A2 and CYP2D6 isotypes. In this experiment, Duloxetine was metabolized using rat microsomes, housing a variety of CYP isotypes. Efficiency of metabolism in the presence and absence of inhibitors was measured using HPLC-MS.

Introduction

For most drugs to be excreted from the body, they must first be inactivated and made more soluble. Cytochrome P450 (abbreviated as “CYP”) isozymes are heme-containing membrane proteins that chemically modify drugs for metabolism, most commonly via oxidation.¹ These proteins are found in the smooth endoplasmic reticulum of cells located in the liver, intestines, lungs, kidneys, plasma, and skin. The main site of drug metabolism in the human body is the liver, therefore, P450 enzyme concentration and activity is highest in hepatic cells.³

CYP enzymes have been categorized using a common nomenclature system that includes the family name, subfamily, and the individual gene that encodes the enzyme. There are more than 30 P450 enzymes that have been identified in human cells and belong to families 1-4. An estimated 90% of drug metabolism has been attributed to six P450 enzymes including CYP1A2, 2C9, 2C19, 2D6, 2E1, 3A4/5 making these enzymes of greatest clinical interest.²

Drug metabolism varies from one person to the next. This is due to the wide variety of P450 enzymes expressed in individuals. These differences in enzyme presence and effectiveness can cause different rates of drug metabolism from one individual to the next and can lead to adverse side effects. Some drugs can act as either CYP activators or inhibitors. CYP activators increase the rate of drug metabolism causing the drug to be excreted at a faster rate. This may cause the effects of the drug to be decreased or lost. Most CYP activators function by increasing expression of CYP enzymes. CYP inhibitors decrease the rate of drug metabolism. This can lead to a toxic buildup of the drug.⁴ Being able to predict and manipulate drug metabolites is useful when designing new drugs to decrease side effects, and can help predict drug-drug interactions when an individual is prescribed multiple drugs that share the same pathway.

In this project, I analyzed the drug metabolites of a soluble antidepressant, duloxetine. Duloxetine is most commonly metabolized by CYP1A2 and CYP2D6, like many other antidepressants. Previous studies have shown a decrease in the production of duloxetine metabolites in the presence of both CYP1A2 and CYP2D6 isozyme specific CYP inhibitors.⁵ Therefore, these specific CYP enzymes are important for metabolism of duloxetine.

Metabolites were produced by incubating duloxetine with rat microsomes containing CYP enzymes of various isotypes. Metabolite production was monitored using HPLC-MS. The efficiency of drug metabolism was manipulated by adding a general cytochrome P450 inhibitor, SKF-525A. SKF-525A is a broad spectrum CYP inhibitor that inhibits CYP activity of all isotypes.⁶ Broad inhibition is expected to show an overall decrease in the percentage of metabolites produced and an increased concentration of duloxetine remaining in the sample. Data was collected using HPLC-MS.

References

1. Groot, M.; Ackland, M.; Horne, V.; Alexander, A.; Jones, B. Novel Approach to Predicting P450-Mediated Drug Metabolism: Developments of a Combined Protein and Pharmacophore Model for CYP2D6. *J. Med. Chem.* **1999**, *42* (20), pp 4062–4070.
2. Di Nardo, G.; Gilardi, G. Optimization of the bacterial cytochrome P450 BM3 system for the production of human drug metabolites. *Int J Mol Sci.* **2012**, *13*(12):15901-24.
3. Cytochrome P450. U.S. National Library of Medicine. **2018**. <https://ghr.nlm.nih.gov/primer/genefamily/cytochromep450>. Accessed 30 November 2018.
4. Patrick, G. L. *An introduction to medicinal chemistry*; Oxford University Press: Oxford, United Kingdom, 2017.
5. Knadler, MP.; Lobo, E.; Chappell, J.; Bergstrom, R. Duloxetine: clinical pharmacokinetics and drug interactions. *Clin Pharmacokinet.* **2011**, *50*(5):281-94.
6. Ortiz, P. 1-Aminobenzotriazole: A Mechanism-Based Cytochrome P450 Inhibitor and Probe of Cytochrome P450 Biology. *Med Chem (Los Angeles)*. **2018**, *8*(3):038.

Materials & Methods

Duloxetine can be metabolized in vitro when incubated with rat microsomes housing a variety of CYP enzymes, with or without an NADPH-generating system. To observe the effects of CYP inhibition on metabolite production a broad spectrum CYP inhibitor, SKF-525A, was added to the incubation mixture.

Rat microsomes, SKF-525A, NADP, G6P, and G6P dehydrogenase were purchased from Sigma-Aldrich and Duloxetine was kindly provided by Dr. Myoung Lee. A 50 mM phosphate buffer (pH 7.4) was used to make a stock solution of Duloxetine with a concentration of 1.6 mM and a stock solution of SKF-525A with a concentration of 1.85 mM. Using these stocks, incubation mixtures were prepared by adding 65 µl of either drug or drug + inhibitor, combined with 25 µl of rat microsomes, and 35 µl of additional buffer. Experimental controls included a sample containing only duloxetine and buffer and a sample with only SKF-525A and buffer. Tubes were incubated for 2 hours. After incubation, 250 µl of methanol was added to stop the reaction. Tubes were centrifuged at top speed for 20 minutes and the supernatant from each tube was extracted and placed in a new tube. The tubes were stored in the freezer until they were run on the HPLC-MS. HPLC separates the components of a mixture and uses absorbance to identify them. Components of the mixture can be ionized and separated based on their mass to charge ratio by the mass spectrometer. A gradient of 5 mM ammonium acetate in water and 5 mM ammonium acetate in acetonitrile and water (95:5) was used. The gradient started at 95% ammonium acetate in water and decreased to 5%. The run time was 35 minutes for each sample. The sample contained 50 µl of the incubation mixture diluted with 1 ml of ammonium acetate in water. The sample was run through the HPLC connected with the mass spectrometer and the type and percentage of each metabolite was determined using SIM and TIC filtered data.

Results

SmartCYP, a freely available online algorithm, was used to predict the metabolites of duloxetine.

| 2D6 Ranking | Atom | 2D6 Score | Energy | 2DSASA |
|-------------|------|-----------|--------|--------|
| 1 | C.13 | 79.5 | 80.8 | 33.5 |
| 2 | C.10 | 79.6 | 74.1 | 28.8 |
| 3 | C.1 | 85.4 | 41.1 | 65.6 |

Figure 1: According to this algorithm the most common metabolites of duloxetine by CYP 2D6 should be hydroxylated and N-demethylated products. We are not able to distinguish between hydroxylated products using the HPLC-MS.

| 3A4 Ranking | Atom | 3A4 Score | Energy | 2DSASA |
|-------------|------|-----------|--------|--------|
| 1 | C.1 | 30.5 | 41.1 | 65.6 |
| 2 | C.3 | 33.6 | 41.1 | 27.1 |
| 3 | N.2 | 46 | 54.1 | 22.4 |

Figure 2: According to this algorithm the most common metabolites of duloxetine by CYP 3A4 should also be hydroxylated and N-demethylated products, but hydroxylation may occur at the nitrogen or amino carbon, rather than an aromatic carbon. This is predicted due to the different size of the CYP 3A4 and 2D6 active sites.

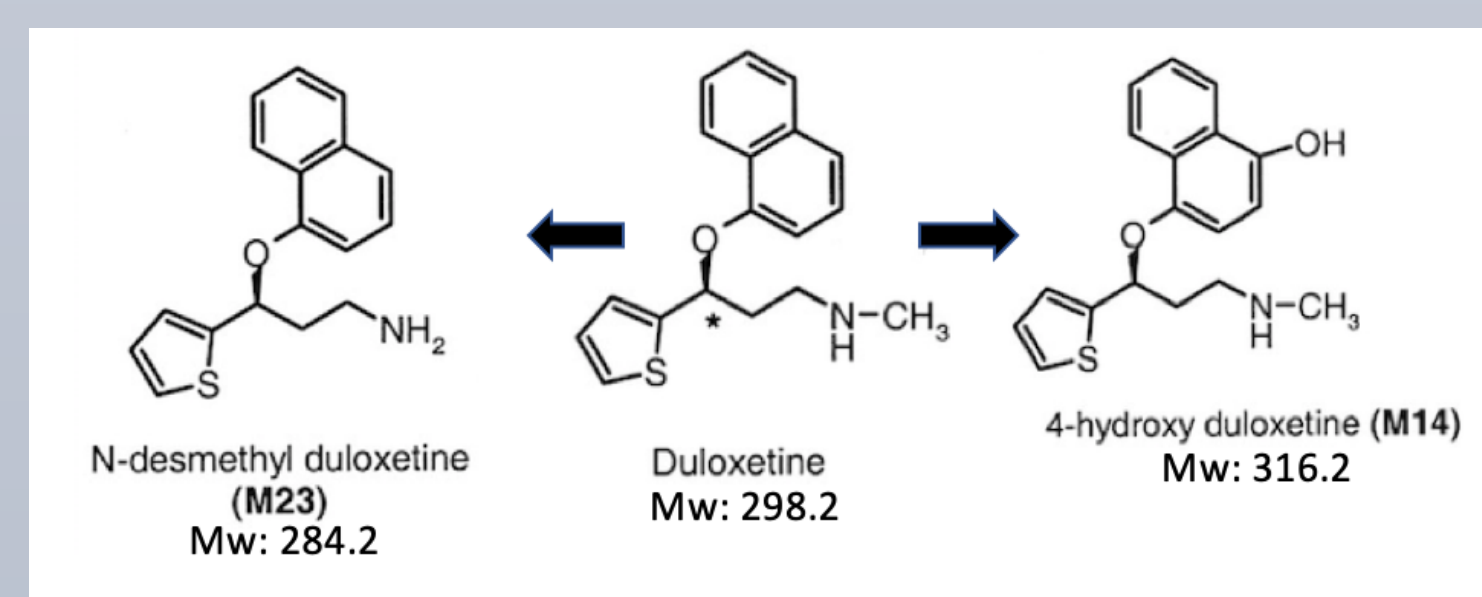


Figure 3: Duloxetine is at the center with its metabolites including N-desmethyl duloxetine and 4-hydroxy duloxetine on either side. Duloxetine with a methyl group removed creates N-desmethyl duloxetine while addition of a hydroxyl group creates 4-hydroxy duloxetine. The molecular weights listed are after polarization of the molecule.

Results Continued

| Trial | Percent of N-desmethyl duloxetine | Percent of unmetabolized duloxetine | Percent of 4-hydroxy duloxetine |
|------------------------------|-----------------------------------|-------------------------------------|---------------------------------|
| Duloxetine without inhibitor | 71.5% | 0% | 28.5% |
| Duloxetine with inhibitor | 65.1% | 10.4% | 24.5% |

Table 1: Percentage of unmetabolized duloxetine and of each metabolite produced was determined using the total ion chromatogram from the mass spectrometer.



Figure 4: Chromatogram of duloxetine after metabolism by CYP enzymes.

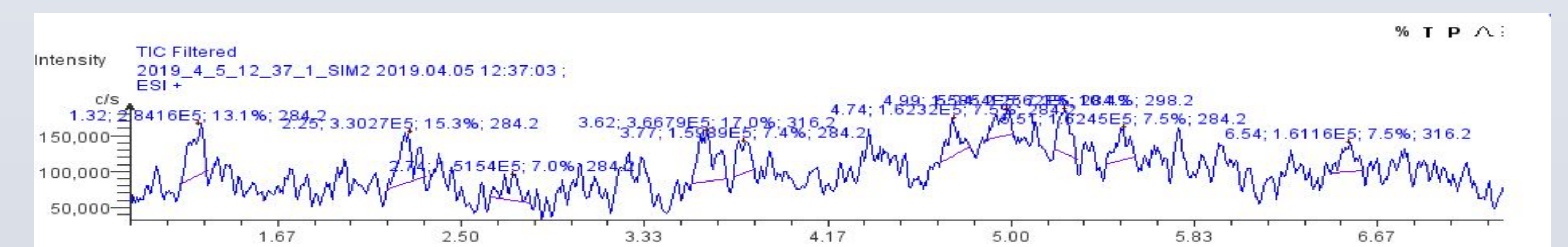


Figure 5: Chromatogram of duloxetine after metabolism by CYP enzymes in the presence of SKF-525A, a general CYP inhibitor.

Discussion

The peaks in the chromatogram were more spread out than expected, and the intensity of the peaks was very low. This may be due to duloxetine and its metabolites sticking to the C18 column, or to product loss at some step in the procedure. Future experiments would aim to get single, more pronounced peaks for each metabolite in the HPLC and mass spectrum data. This could possibly be achieved by modifying the gradient parameters on the HPLC, using one single solvent instead of a gradient, or injecting the sample directly into the mass spectrometer instead of using the HPLC-MS combined.

By analyzing the total ion chromatogram, we find that duloxetine without the inhibitor showed 100% metabolism. Metabolism was reduced by 10% in the presence of CYP inhibitor, SKF-525A. In both cases metabolism was high, most likely because there was a high concentration of rat microsomes added to both samples. Previous studies, completed by Dani Schmaus, diluted the microsomes with phosphate buffer. For this experiment, 25 µl of undiluted rat microsomes were added to the incubation mixture, producing very high rates of metabolism. Another manipulation to the protocol was the removal of the NADPH-generating system. Previous research also showed the NADPH-generating system was not necessary to metabolize duloxetine. Removal of this system reduced the cost of the experiment, eliminated steps to create the NADPH-generating system from the procedure, as well as cleaned up the sample overall. An increase in CYP inhibition and metabolite production could be seen in future experiments if the concentration of SKF-525A was increased, rat microsome concentration was decreased, or isotype specific CYP inhibitors were used.

Acknowledgments

This project was funded by Winona State Universities Undergraduate Research and Creative Project Grant. I want to give a special thanks to my research advisor, Dr. Emily F. Ruff, research advisor, Dr. Myoung E. Lee, and previous research student, Dani Schmaus.